PB80-224249

Identification and Detection of Water-Borne Viruses by Immunoenzymatic Methods

Harvard School of Public Health
Boston, MA

Prepared for

Environmental Monitoring and Support Lab.
Cincinnati, OH

Jun 80

U.S. Department of Commerce
National Technical Information Service
NTIS.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/4-80-034		PB80-224249

4. TITLE AND SUBTITLE	5. REPORT DATE
Identification and Detection of Water-Borne Viruses by Immunoenzymatic Methods	JUNE 1980 ISSUING DATE.
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
John E. Herrmann, Ph.D.	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Harvard School of Public Health Boston, MA 02115	A38B1D
	11. CONTRACT/GRANT NO.
	R-803360
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Environmental Monitoring and Support Lab.-Cincinnati Office of Research and Development U. S. Environmental Protection Agency Cincinnati, OH 45268	Final 4/75-3/80
	14. SPONSORING AGENCY CODE
	EPA/600/06

15. SUPPLEMENTARY NOTES

Project Officer: Dr. Robert S. Safferman (513) 684-7334

16. ABSTRACT

A quantitative enzyme-linked immunosorbent assay (ELISA) was used for identification of viruses selected as representative water-borne viruses: poliovirus 1, echovirus 6, coxsackievirus A9, and coxsackie B viruses. Partially purified viral antigens or virus-specific antibodies were adsorbed to polystyrene spectrophotometer cuvettes, which permitted the assays to be reported and compared in terms of enzyme units specifically reacting. Inhibitors in diluents used to prevent non-specific adsorption of immunoreagents caused desorption of virus or antibody during an immunoassay; the amount of virus desorption varied with the type of preparation used, and antibody desorption was dependent on the concentration of antibody initially adsorbed. For specific identification of a given enterovirus type by this ELISA method, approximately 10^5 plaque-forming units of virus per assay tube were required. To alleviate the problem of antibody and virus desorption, antibodies and virus were immobilized by covalent linkage on nylon balls for use in solid-phase enzyme-linked immunoassays. A higher percentage of virus could be immobilized by this method than was possible by adsorption to polystyrene, and enzyme-linked immunoassay on nylon was sufficiently specific to differentiate the three poliovirus types.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Viruses,* water, wastewater, immunoassay	Detection, identification, monitoring	06M

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to public	Unclassified	44
	20. SECURITY CLASS *(This page)*	22. PRICE
	Unclassified	

EPA Form 2220-1 (9-73)

☆ U.S. GOVERNMENT PRINTING OFFICE: 1980--657-165/0003

United States
Environmental Protection
Agency

Environmental Monitoring and Support
Laboratory
Cincinnati OH 45268

EPA-600/4-80-034
June 1980

Research and Development

PB80-224249

EPA

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EPA-600/4-80-034
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IDENTIFICATION AND DETECTION OF WATER-BORNE VIRUSES BY IMMUNOENZYMATIC METHODS

by

John E. Herrmann
Harvard School of Public Health
Boston, Massachusetts 02115

Grant No. R-803360

Project Officer

Robert S. Safferman
Biological Methods Branch
Environmental Monitoring and Support Laboratory
Cincinnati, Ohio 45268

ENVIRONMENTAL MONITORING AND SUPPORT LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

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FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of waste effluents. The Environmental Monitoring and Support Laboratory - Cincinnati, conducts research to:

Develop and evaluate techniques to measure the presence and concentration of physical, chemical, and radiological pollutants in water, wastewater, bottom sediments, and solid waste.

Investigate methods for the concentration, recovery, and identification of viruses, bacteria and other microbiological organisms in water; and to determine the responses of aquatic organisms to water quality.

Develop and operate an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.

Develop and operate a computerized system for instrument automation leading to improved data collection, analysis, and quality control.

This research was conducted in the interest of applying immunoenzymatic techniques to the identification of enteroviruses isolated from varying qualities of waters including sewage, river, sea and tap water. Such methodology may bring about significant improvement over techniques currently in use in the detection and identification of waterborne viruses.

Dwight G. Ballinger
Director
Environmental Monitoring and Support
Laboratory - Cincinnati

ABSTRACT

A quantitative enzyme-linked immunosorbent assay (ELISA) was used for identification of viruses selected as representative water-borne viruses: poliovirus 1, echovirus 6, coxsackievirus A9, and coxsackie B viruses. Partially purified viral antigens or virus-specific antibodies were adsorbed to polystyrene spectrophotometer cuvettes, which permitted the assays to be reported and compared in terms of enzyme units specifically reacting. Both the adsorbed antigen and adsorbed antibody methods were approximately equal in terms of sensitivity and specificity of reaction. By use of ^{14}C-leucine-labelled enteroviruses, the amount of virus that binds to the plastics used was dependent on the purity of the virus preparation used, but was higher than the amount that was bound by plastics coated with viral antibody. It was also shown that the inhibitors in diluents used to prevent non-specific adsorption of immunoreagents caused desorption of virus or antibody during an immunoassay; the amount of virus desorption varied with the type of preparation used, and antibody desorption was dependent on the concentration of antibody initially adsorbed. For specific identification of a given enterovirus type by this ELISA method, approximately 10^5 plaque-forming units of virus per assay tube were required. To alleviate the problem of antibody and virus desorption, and to increase the amount of each that could be bound to plastics, antibodies and virus were immobilized by covalent linkage on nylon balls for use in solid-phase enzyme-linked immunoassays. Covalent linkage of antibody or virus to nylon was accomplished by treatment of partially hydrolized nylon with glutaraldehyde or carbodiimides. Up to 0.74 µg of immunoglobulin G per mm^2 nylon could be immobilized, whereas only 0.02 µg per mm^2 could be adsorbed to polystyrene, and the binding to nylon was stable. This eliminated the problem of antibody desorption and gave more reproducible results. Also, antibodies coupled to nylon balls remained bound under conditions that dissociate antibody-antigen complexes, which permitted reuse of the immobilized antibodies for immunoassays. A higher percentage of virus could be immobilized by this method than was possible by adsorption to polystyrene, and enzyme-linked immunoassay on nylon was sufficiently specific to differentiate the three poliovirus types.

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SECTION 1

INTRODUCTION

There are approximately 100 enteric viruses that infect humans, including those in the enterovirus group, adenoviruses, reoviruses, hepatitis agents, and gastroenteritis agents. Many of them are highly virulent, and cause a wide variety of clinical diseases. Despite concern for these viruses, there is as yet no rapid practical assay for their identification and detection, which is limited to clinical diagnosis, epidemiological studies on their occurrence and impact, and their control. The purpose of the present study was to develop immunochemical methods which would be applicable to rapid viral diagnosis. The methods we concentrated on were solid-phase enzyme-linked immunoassays, and we selected enteroviruses as model water-borne viruses to be assayed.

Solid phase enzyme immunoassays, usually called enzyme-linked immunosorbent assays (ELISA, ref. 1), and also enzyme-linked immunospecific assays (2), have been applied to the detection of antibodies to a variety of microbial and parasitic infections, as well as to toxin detection, hormone assay, and a number of other chemical substances. The application of the method to virus identification is more limited, but has been used to identify plant viruses (3), hepatitis B antigen (4), hepatitis A antigen (5), herpesviruses (6), and human reovirus-like agent (7).

For enterovirus identification, the most common method used is virus neutralization which, because of the large number of enterovirus types can be both time-consuming and costly. Immunofluorescent (8,9,10) and immunoperoxidase techniques (11) for enterovirus identification have been described, but require subjective judgements which are always a factor in histochemical tests. For this reason, the suitability of enzyme-linked immunoassay for use in enterovirus identification was investigated. The present report describes the identification of selected enterovirus types by this technique, the factors involved in the assay, and the development of an enzyme-linked immunoassay based on coupling of antibodies and viral antigens to nylon. The latter aspect was undertaken because the ELISA method, which is based on simple adsorption of antigens or antibodies to plastics, is limited by the amounts of antigen or antibody that can be adsorbed (2,3) to a given plastic, and the problem of elution of what is adsorbed during an immunoassay procedure (2). Lack of reproducibility is also a problem, and antisera adsorbed cannot be re-used.

To alleviate this problem, more efficient solid-phase supports were sought, utilizing some of the technology developed for preparing immobilized

enzymes. Among the supports used for enzyme immobilization have been various types of nylon, which chemically resemble polypeptides in structure. Activation of nylon tubing by chemical treatments permits it to be coupled covalently to enzymes (14,15), and activated nylon fibers have been used as immunosorbents for cell fractionation (16). Treatment of nylon with HCl for limited periods of time causes partial hydrolysis, which results in free amino and carboxyl groups. Thus, proteins can be covalently coupled through either group by use of the appropriate bifunctional reagents. We adapted this to our purposes, namely the coupling of antibodies and virus antigens to solid-phase carriers for immunoassay, by use of chemically activated nylon balls. These methods were compared with methods based on adsorption for both immobilization of virus antigens and antibodies, and for usefulness in enzyme-linked immunoassay of enteroviruses.

SECTION 2

CONCLUSIONS

The methods developed were designed for identification of enteroviruses but should be applicable to other water-borne viruses as well. In terms of identification, enzyme-linked immunoassays based on either adsorption to polystyrene or covalent linking to nylon were satisfactory for identifying selected enterovirus types. The limitations of the method appear to be related to the specificity of the viral antisera available. Both individual sera and serum pools are satisfactory for typing enteroviruses by virus neutralization, but all are not satisfactory for use in immunochemical studies. This appears to be due in part to antibody against cell antigens, but also appears to be due to spurious cross-reactions of undetermined origin.

In terms of sensitivity, the amount of antigen needed to give a positive reaction by enzyme-linked immunoassays developed to date is approximately lng. For the enterovirus we tested, 10^5 plaque-forming units of virus were needed per assay. Although this amount is readily available from infected tissue cultures, it is well above the amount that would be found in water. Use of radioactive enzyme substrates have been reported to increase the sensitivity of enzyme-linked immunoassays by up to 1000 times (17), which would thus give a sensitivity of 100 plaque-forming units per assay, utilizing antisera currently available. Preparation of antisera that is immunospecific and of high titer could possibly increase the sensitivity another order of magnitude, which would allow for direct detection of virus in at least some water samples which have been processed and concentrated for virus detection.

SECTION 3

RECOMMENDATIONS

Enzyme-linked immunoassays have been found useful for identification of a number of viral antigens, including those in the enterovirus group reported here. The limitations on the method for type-specific identification of all enterovirus types appears to be the specificity, for immunochemical work, of the antisera that is readily available to all investigators. Because of this, it is recommended that sera be prepared from purified enterovirus virions, and subsequently tested for type-specificity by immunochemical methods. For use as a detection method for viruses in water, the maximum sensitivity that would appear to be obtainable by current technology is 100 infectious units of virus per assay. This is close to the amount found in some concentrated water samples and the sensitivity could undoubtedly be improved by use of high titer, immunospecific viral antisera. Although preparation of such antisera for all the virus types considered to be water-borne would be an extensive project, preparation of antisera for a few selected types most frequently isolated would not be difficult. This might permit a pilot study to be made for direct testing of these virus types in concentrated water samples and in waste water.

SECTION 4

MATERIALS AND METHODS

VIRUSES AND TISSUE CULTURES

Poliovirus types 1-3, echovirus type 6, coxsackievirus B types 1, 4-6, and coxsackievirus A type 9 were obtained from the NIH Research Resources Branch, Bethesda, Maryland. Coxsackievirus types B2 and B3 were obtained from D.O. Cliver, University of Wisconsin, Madison. Viruses were propagated in vero cells (obtained from D.O. Cliver) or in BGM cells (obtained from R.S. Safferman, Environmental Protection Agency, Cincinnati, Ohio).

VIRUS ANTIGENS

Virus was innoculated onto cell monolayers in 75 cm^2 flasks and incubated with serum-free MEM medium, 5 ml per flask. After the cells showed 3 to 4 + cytopathic effects, they were frozen-thawed twice. Cell debris was removed by centrifugation at 40,000 xg (Sorvall RC-2B) for 30 min. Five grams of anion exchanger (Bio Rad AG2-X8) was washed in distilled water and added to 30 ml virus solution. The mixture was stirred with an overhead stirrer at 300 rev/min for 1 h and filtered through a Millipore fritted-glass filter. By plaque titration, no virus losses were noted by use of the anion-exchange resin or the filtration step. Uninfected cells were processed in the same way for use as cell antigen controls.

Virus antigens were also prepared by extraction with diethyl ether. Virus suspensions clarified by centrifugation as above were mixed with an equal volume of cold (4°C) ether, and held on ice for 2h. The aqueous layer was collected, and the residual ether removed under vacuum.

RADIOACTIVE VIRUS

For preparation of ^{14}C-leucine-labelled poliovirus 2 and coxsackievirus B3, BGM or vero cultures were starved of leucine for 18h by incubation with Earle's balanced salt solution (EBSS). The cultures were inoculated with virus suspended in EBSS at a concentration of ca. 10 plaque forming units (PFU) per cell, adsorbed 30 min at room temperature, and rinsed twice with EBSS. Uniformly labeled ^{14}C-leucine (specific activity 270 mCi/mM), (New England Nuclear, Boston, Mass.) in EBSS (20 µCi/ml) was added with or without 1 µg/ml Actinomycin D, and the cultures incubated for 24 h at 37°C. The cultures were freeze-thawed three times, and the harvested virus centrifuged at 2500 x g to remove cell debris. The supernatant fluids were collected and the labelled virus purified (18) by passage through a DEAE-

Sephadex A-50 column (1.5 x 30 cm), using 0.06M phosphate buffer, pH7.5, as eluent. The effluent column fractions in which peak counts/min (Searle Model 6880 Liquid Scintillation Counter) and peak virus PFU coincided were the fractions used as labelled virus.

IMMUNOGLOBULINS AND ANTISERA

Rabbit IgG was obtained from Miles Laboratories, Elkhart, IN. Goat anti-rabbit IgG sera was obtained from Antibodies, Inc., Davis, CA. The IgG fractions of all sera were prepared by $(NH_4)_2SO_4$ precipitation at one-third saturation, followed by dialysis against 0.015M phosphate-buffered saline, pH7.2 (PBS).

Viral antisera used were horse antiviral sera obtained from the NIH Research Resource Branch, Bethesda, Maryland, or rabbit antiviral sera obtained from Microbiological Associates (Bethesda, Maryland). For use in the assays, viral antisera were absorbed with cell debris prior to use. This was done by freeze-thawing cell cultures, centrifuging the cell debris at 2500 x g, and resuspending the pellet (0.1 ml) in ml of antisera diluted 1:10 PBS plus 1% bovine serum albumin (BSA). The cell-sera mixture was incubated 24 h at 4°C, and the cell debris removed by centrifugation at 2500 x g. Sera were tested for absence of antibody to cell antigens by enzyme immunoassay prior to use.

IODINATED IgA

IgA, (mouse myeloma protein), obtained from G. Kelsoe, Harvard University, was labelled with ^{125}I by use of the method described by Bolton and Hunter (19). The preparation used had approximated 3×10^5 counts/min/ng protein.

ENZYME-LABELLED ANTIBODIES

Peroxidase from horseradish (E.C. 1.11.1.7), Sigma type VI, Sigma Chemical Co., St. Louis, MO, sp. act. 274 units/mg, was coupled to the IgG fraction of goat anti-rabbit IgG or rabbit anti-horse IgG by use of periodate (20). Peroxidase-labelled globulin was separated from unlabelled material by gel filtration on 2.5 x 80 cm columns of Sepharose 6B. Fractions that gave maximum absorption in a spectrophotometer at both 403 nm (enzyme) and 280 nm (protein) were pooled and precipitated by addition of $(NH_4)_2SO_4$ to 40% saturation. The precipitate was suspended in a volume of PBS to give a protein concentration of approximately 2 mg/ml, and dialysed against PBS for 3 days at 4°C. The preparations were tested for immunologic reactivity in gel-diffusion plates against the appropriate globulin, and stored at -20°C until used.

ENZYME IMMUNOASSAY

The procedure for the conventional solid-phase enzyme-linked immunoassay was based on the ELISA of Engvall and Perlman (1), as modified by Ruitenberg, et al. (21). Virus antigens diluted 1:4 in PBS were added to wells of polystyrene Microtiter plates (Cooke Engineering, Alexandria,

Virginia), 0.1 ml/well, or to polystyrene spectrophotometer cuvettes (Variable Volumetrics, Inc., Woburn, Mass.), 0.2 ml/cuvette. Both plates and cuvettes were pre-treated with 25 µg/ml poly-L-Lysine in PBS (22) to enhance antigen binding. The antigens were adsorbed 1 h at 37°C, plus overnight at 4°C.

The plates or cuvettes were washed three times with distilled water or PBS plus 0.05% (v/v) Tween 20. Dilutions of test and control sera were added (0.05 ml/well, 0.1 ml/cuvette) and incubated 30 min at 37°C. The samples were washed as above and peroxidase-conjugated antiglobulin added (0.05 ml/well, 0.1 ml/cuvette) at a 1:100 dilution. The diluent for the sera and the conjugates was PBS with 2% w/v BSA and 0.15% (v/v) Tween 20 added. Optimal dilutions of viral antigens and immunoreagents used were determined by "checkerboard" titrations. For the Microtiter plate assay the plates were incubated 30 min at 37°C, washed as above, and 0.2 ml/well substrate (0.05% 5-amino salicylic acid in distilled water adjusted to pH 6.0 with 1N NaOH, plus 0.005% H_2O_2) added plus one drop of 1% w/v gelatin to help prevent precipitation of the reaction product. After 30 to 60 min at room temperature, the reaction was stopped with one drop of 1.5M sodium azide. A red-brown reaction product is formed; the end points were read visually by comparison with controls (antigen plus normal serum and/or heterotypic viral antisera, and antigen plus PBS).

For spectrophotometric assays, the cuvettes were incubated at 37°C for 30 min, washed as above, 0.1 ml of phosphate buffer, 0.01M, pH 6 added and 2.9 ml of enzyme substrate (3×10^{-4}M O-dianisidine dihydrochloride, 0.001M H_2O_2) in the same buffer added. The reaction was monitored (A_{460}) with a Zeiss PM6-KS recording spectrophotometer and enzyme units calculated. One unit of peroxidase is the amount of enzyme decomposing 1 µ mol of H_2O_2 per min at 25°C under the conditions above.

For comparison to the above method, an adsorbed antibody method was also used. Virus antisera (horse) at a 1:50 dilution were adsorbed to cuvettes as above. After adsorption and washing the samples, ether-extracted virus preparations diluted 1:4 in PBS plus 2% BSA and 0.15% Tween 20 were added (0.1 ml/cuvette), and incubated 1.5 h at room temperature. The samples were washed with PBS-Tween, 0.1 ml diluted rabbit antiviral sera (1:100) added, and incubated 30 min at 37°C. Subsequent treatment and addition of conjugates and enzyme substrates were the same as that for the adsorbed antigen method above. In addition to the use of poly-L-lysine to enhance antibody binding to plastics, glutaraldehyde was also tested, as described (23).

ACTIVATED NYLON

Nylon 6/6 (poly-hexamethylene-adipamide) balls (Precision Plastic Ball Co., Chicago, IL), 3.2mm diameter, were incubated 30 min in 3.5M HCl. The balls were washed in distilled water, treated with concentrated acetic anhydride 1 min, washed in distilled water, washed 1 min in 0.1M carbonate buffer (pH9.5), washed in phosphate-buffered saline and reacted with either 1-cyclohexyl-3- (2-morpholinoethyl) -carbodiimide metho-p-toluene sulfonate (CMC), 4% v/v in distilled water for 10 min, or with glutaraldehyde (acetic

anhydride treatment omitted), 8% v/v in distilled water, for 2 h. All procedures were done at room temperature.

Activated nylon powder was also prepared for use in antibody immobilization. This was done by dissolving nylon 6/6 in conc. HCl (approx. 1g nylon per 5ml HCl). The dissolved nylon was precipitated as powder (particles of approx. 1 µm diameter) by dropwise addition to distilled water at room temperature. The precipitate was washed by centrifugation at 3500 X g in 0.1M sodium carbonate buffer (pH9.5), washed in distilled water, and activated for coupling by incubation with glutaraldehyde, as above. The effectiveness of the HCl hydrolysis procedure in exposing free amino groups on nylon balls and powder was monitored by a color reaction with 2,4,6-trinitrobenzenesulfonic acid, used as described for determination of amines (24).

COUPLING OF ANTIBODY AND VIRUS TO NYLON

The activated nylon balls or powder were washed in distilled water and incubated with IgG fractions of antisera, normal sera or virus solutions for 2 h at room temperature plus 18 h at 4°C for CMC-activated nylon and 4 h at room temperature for glutaraldehyde-activated nylon. IgG solutions used for coupling were diluted in PBS at concentrations of approx. 100 µg/ml. Virus was diluted as for the conventional ELISA tests above. The nylon was washed in PBS, and a 2% w/v solution of BSA in PBS added for 1 h to bind unreacted CMC or aldehyde groups.

In some experiments, chemical spacers were added to the modified nylon to lessen steric hindrance of immobilized antibody. For this purpose, nylon balls were treated with 3.5M HCl as above and rinsed with phosphate-buffered saline several times, until the pH of the mixture was 7.0. Poly-L-Lysine, 1 mg/ml, in phosphate-buffered saline, was added followed by a 4% v/v solution of CMC in the same buffer. The nylon was rinsed in phosphate-buffered saline, 4% v/v glutaraldehyde was added, and incubated 30 min at room temperature. The balls were rinsed again, and solutions of antibody added, as above.

ENZYME-LINKED IMMUNOASSAY ON NYLON

The method used for enzyme-linked immunoassay of antibody on nylon was similar to that as described for the conventional ELISA tests above. Four to 6 activated nylon balls were placed in 12 x 75mm glass tubes and IgG fractions of antiglobulin coupled to them as described above. Globulin, diluted in PBS with 2% w/v BSA and 0.05% v/v Tween 20, was added (0.5 ml/tube) and incubated 1 h at 37°C. The balls were washed, and 0.25ml of peroxidase-labeled antiglobulin, diluted 1:100 in the same diluent as that used for globulin, added. After 30 min at 37°C, the balls were washed with PBS with 0.05% v/v Tween 20 added, and removed to cuvettes. Enzyme substrate (1.5 ml/cuvette of5-amino-salicylic acid plus H_2O_2, ref. 21) was added. After 15 min incubation at room temperature, the reaction was stopped with 0.1 ml of 1.5M NaN_3, and the absorbance at 460mm determined. For comparison to the above, a conventional ELISA test was done utilizing antibody adsorbed to 3.2mm polystyrene balls. All assays were done in duplicate, and recorded

as mean values.

Two methods were used for enzyme-linked immunoassay on nylon for enteroviruses, the coupled antigen and coupled antibody methods. The general procedure for the coupled antigen method was to 1) react viral antigens with activated nylon balls, 2) add antiviral sera, 3) add enzyme-labelled antiglobulin, 4) add enzyme substrate and read reactions. Viral antigens were either unpurified virus (fluid from infected cell cultures) or partially purified virus. Virus antigens (purified or unpurified) were diluted 1:4 in PBS and immobilized on activated nylon balls as described. Four to six balls were placed in 12 x 75mm tubes, and dilutions of virus antiserum in PBS with 2% w/v BSA and 0.15% Tween 20 added (0.5 ml/tube). The tubes were incubated at 37°C, 30 minutes, washed as above, and 0.25 ml of peroxidase-conjugated antiglobulin added. The conjugate was diluted 1:100 in PBS with 2% BSA and 0.15% Tween 20 added. After 30 min at 37°C, the balls were washed 3 times with PBS plus 0.05% Tween 20, and removed to cuvettes. Enzyme substrate was added and the reactions monitored as above.

The general procedure for the coupled antibody method was to 1) react viral antibody with activated nylon balls, 2) add viral antigen, 3) add antiviral sera, 4) add enzyme-conjugated antiglobulin. When used as an indirect test, it was necessary to use antiviral sera prepared in two different species of animals. IgG fractions of viral antisera (horse) diluted in PBS were coupled to nylon balls as described. Viral antigen, diluted 1:4 in PBS was added (0.5 ml per 4 balls, in 12 x 75 mm tubes) and incubated for 30 min. at room temperature plus overnight at 4°C. The balls were washed 3 times with PBS plus 0.05% Tween 20, and incubated with viral antisera rabbit, diluted in PBS. The procedure from this point on followed that described just above. The enzyme conjugate used was directed against the rabbit antisera.

SECTION 5

RESULTS

VIRUS PURIFICATION PROCEDURES

Because there is a limited amount of protein that can be adsorbed to plastics (12,13), viral antigens were partially purified. The efficiency of the virus purification procedures for removing cellular protein was tested with uninfected BGM cell cultures, treated in the same manner as virus-infected ones. The amount of protein removed after each treatment with Bio Rad AG 2X8 anion exchange resin, or by ether extraction was measured by absorption at 280 nm and the method of Lowry, et al. (25). The protein standard used for comparison was bovine serum albumin (BSA). It can be seen from the results shown in Table 1 that one extraction with ether removed as much protein as one ion-exchange treatment. Subsequent ether extractions (not shown) did not give additional protein reduction, but further ion-exchange treatments did. The same extraction procedures used for virus preparations did not cause any loss of virus as measured by the plaque method. For use in enzyme immunoassay tests below based on adsorption to plastics, one ether extraction or two anion-exchange treatments proved to be sufficient.

TABLE 1. VIRUS ANTIGEN PURIFICATION PROCEDURE: REMOVAL OF CELLULAR PROTEIN

Procedure	Protein concentration (mg/ml)	Protein reduction (%)
Centrifugation*	0.61	(0)
Ion-exchange, extraction 1	0.42	31
2	0.26	57
3	0.15	75
Ether extraction	0.38	38

*Cells removed from suspension by centrifugation at 40,000 x g for 30 min.

NON-SPECIFIC ADSORPTION

To test for non-specific adsorption of immunoreagents to plastics, peroxidase-labeled anti-rabbit globulin was diluted in PBS with varying amounts of BSA and Tween 20 added and adsorbed to spectrophotometer cuvettes (1 h at 37°C). The substrate addition and reading of results were as described for enzyme immunoassay tests above.

The results in Table 2 show that at lower concentrations of BSA and Tween 20 that some peroxidase-labeled antiglobulin adsorbs to polystyrene spectrophotometer cuvettes non-specifically. Concentrations of Tween 20 at 0.15% (v/v) plus BSA at 2% (w/v) gave about as low a background as any combination tried. Also, these concentrations did not interfere with antigen-antibody reactions, as measured by precipitin tests and enzyme immunoassay tests. Thus, we selected this combination as a standard diluent (PBS-BSAT diluent) for both viral antibody and peroxidase-labeled antiglobulin.

TABLE 2. INHIBITION OF NON-SPECIFIC ADSORPTION OF IMMUNOREAGENTS

Inhibitor*		Enzyme conjugate adsorbed (Enzyme units)
Bovine serum albumin (% w/v)	Tween 20 (% v/v)	
0	0	0.50
1		0.16
2		0.08
3		0.11
4		0.06
0	0	0.58
	0.05	0.28
	0.10	0.04
	0.15	0.02
	0.20	0.02
2	0	0.08
	0.05	0.03
	0.10	0.02

(continued)

TABLE 2. (continued)

Inhibitor*		Enzyme conjugate adsorbed (Enzyme units)
Bovine serum albumin (% w/v)	Tween 20 (% v/v)	
	0.15	0.01
	0.20	0.01

*Inhibitors added to PBS in the amounts indicated.

ANTIBODY ADSORPTION

To determine the effect of antibody concentration on adsorption to polystyrene, and to find if the above diluent would cause elution of antibody adsorbed to plastics, rabbit anti-horse IgG labeled with peroxidase was added to normal rabbit IgG to give final IgG concentrations of 2, 10, and 100 µg/ml. These were adsorbed to polystyrene tubes in the same manner as that which is used for an immunoassay (1 h at 37°C plus overnight at 4°C). The tubes were incubated for 1 h at 37°C with diluents (PBS with BSA and/or Tween 20), washed, and enzyme substrate added. The product was determined spectrophotometrically, and the number of enzyme units bound to the tubes calculated. The results are shown in Table 3. It can be seen from the table that the diluent used did not cause elution of adsorbed antibody at 10 µg/ml. This could cause some loss of sensitivity if the serum antibody to be adsorbed needed to be used at a low dilution (<1:100), assuming an IgG level of 10 mg/ml in serum. Prior treatment of the tubes with BSA and glutaraldehyde (23) did not increase the number of enzyme units that could be adsorbed.

TABLE 3. ADSORPTION OF PEROXIDASE-LABELED ANTIBODY TO POLYSTYRENE TUBES

PBS diluent added		Enzyme units bound (%)*		
		Antibody protein concentration (µg/ml)		
BSA (%)	Tween-20 (%)	2	10	100
0	0	42.6	44.4	47.2
	0.15	38.5	42.8	27.1
1	0	38.5	50.1	37.3
	0.15	38.1	47.6	30.1
2	0	30.9	49.0	33.1

(continued)

TABLE 3. (continued)

PBS diluent added		Enzyme units bound (%)*		
		Antibody protein concentration (µg/ml)		
BSA (%)	Tween-20 (%)	2	10	100
	0.15	31.7	41.5	29.0

*Based on the number of units bound/number of units in adsorbing solutions, after treating tubes coated with peroxidase-labeled antibody for 1 h at 37°C with the diluent indicated.

ADSORPTION OF ENTEROVIRUSES

The sensitivity of a solid-phase immunoassay depends in part on the amount of antigen adsorbed to the solid-phase surface, or to an antibody-coated surface. The amount of enterovirus that adsorbs was determined by use of ^{14}C-leucine labeled coxsackievirus type B3(CB-3) and/or ^{14}C-leucine labeled poliovirus type 2 (PO-2). For direct antigen adsorption, labeled virus at a 1:4 dilution in PBS was adsorbed to polystyrene tubes, as described in Materials and Methods for enzyme immunoassay, and either tested for virus adsorbed directly, or incubated with PBS-BSAT diluent prior to assay. The bottoms of the tubes were cut off, dissolved in scintillation fluid, and counted. It can be seen from the results shown in Table 4 that both types of labeled virus, in which >95% of the radioactivity is virus associated (26) when purified by column chromatography, adsorbed better when used alone than when paritally purified (batch method ion-exchange resin) unlabeled virus was added. This might be due to the relatively high amounts (ca. 250 µg/ml) of cellular protein present in unlabeled preparations. For both types of viruses and preparations used, there was considerable elution of adsorbed virus by incubation with PBS-BSAT diluent. Based on the initial infectivity titer (PFU/ml) of input virus used, the maximum amount remaining adsorbed in unlabeled preparations would be 1.0×10^6 PFU/tube for PO-2, and 8.1×10^5 PFU/tube for CB-3. Virus also adsorbed to the glass tubes used as controls. There was less initial adsorption for most samples but what did adsorb was less readily eluted.

TABLE 4. ADSORPTION OF ^{14}C-LEUCINE-LABELED ENTEROVIRUSES TO POLYSTYRENE TUBES

Virus preparation	Virus adsorbed (%)*					
	polystyrene		treated polystyrene†			
	Initial (I)‡	Eluted (E)§	I	E	I	E
^{14}C-labeled PO-2	65.5	30.8	64.5	40.85	29.1	28.9

(continued)

TABLE 4. (continued)

Virus preparation	Virus adsorbed (%)*					
	polystyrene		treated polystyrene†			
	Initial (I)‡	Eluted (E)§	I	E	I	E
^{14}C-labeled PO-2 + PO-2[e]	11.8	5.5	11.6	6.1	18.3	14.3
^{14}C-labeled CB-3	65.9	34.2	68.6	38.0	32.7	23.9
^{14}C-labeled CB-3 + CB-3#	20.0	6.3	24.1	8.1	17.0	15.4

*Counts per min (cpm)/cpm of input virus, x 100.

†Tubes treated with poly-L-lysine, as described in Methods.

‡Percent of input cpm adsorbed to tubes after three washes with PBS plus 0.05% Tween 20.

§Percent of input cpm adsorbed to tubes after incubation for 1 hr at 37°C with PBS plus 0.15% Tween 20 and 2% BSA, and washing as above.

#^{14}C-leucine-labeled enteroviruses plus unlabeled enterovirus at the concentration used for immunoassays.

For comparison to the direct adsorption of virus to plastics above, the uptake of virus by antibody coated tubes was measured. Labeled CB-3 and labeled plus unlabeled CB-3 were diluted as above in PBS-BSAT diluent, incubated for 1.5 h at room temperature, washed, and the tubes assayed for bound cpm. The results presented in Table 5 show that the maximum amount of virus uptake was 5.2% of input virus for labeled virus alone, and 4.1% of labeled plus unlabeled virus. Thus, the amount of virus available for immunoassay is approximately the same as that obtained by direct adsorption above for unlabeled plus labeled virus, but is less for the labeled virus alone. The data also show that because there was only a slight increase in uptake of the labeled virus alone, purity of virus, i.e., absence of cell protein, is not as important a factor in this type of assay, which is based on an immune reaction for virus binding.

TABLE 5. BINDING OF ^{14}C-LEUCINE LABELED COXSACKIEVIRUS TYPE B3(CB-3) BY ANTIBODY COATED TUBES

Virus preparation	Anti-CB-3 dilution adsorbed	Cpm bound (%)*
^{14}C-labeled CB-3	1:10	1.4
	1:50	5.2
	1:100	1.5
	none	0.2
^{14}C-labeled CB-3 +CB-3†	1:10	1.2
	1:50	4.1
	1:100	0.9
	none	0.5

*Counts per min (cpm) bound to tubes/cpm of input virus, x100.

†^{14}C-leucine labeled CB-3 plus unlabeled CB-3 at the concentration used for immunoassay.

Use of glutaraldehyde (data not shown), used either directly or in conjunction with adsorbed BSA, did not increase the uptake of labeled virus by either of the two methods used.

VIRUS IDENTIFICATION

The Microtiter plate method was used as a preliminary test to insure that viral antisera did not visibly react with cellular antigens, to demonstrate reactivity of viral antisera with specific virus types, and to determine the optimal dilutions of viral antigens and immunoreagents. The spectrophotometric assay, which yields quantitative data, was used for the virus identifications reported here. Enzyme units bound were calculated for both positive (type-specific) sera and for control sera.

To determine the positive/negative (P/N) ratio required for these assays to be considered positive, an enzyme-linked immunoassay for coxsackievirus type B2 (CB-2) was used. CB-2 antigen was adsorbed to polystyrene cuvettes in replicate samples; sera used were rabbit anti-CB2 and

normal rabbit serum both diluted 1:100 in PBSA-T. The indicator of the reaction was peroxidase-labeled goat anti-rabbit IgG. The data obtained are shown in Table 6. From these data, it was calculated by the Mann-Whitney test that the two groups of values represent distinct populations at the 95% confidence level, and that the difference between a test and control sera would need to be 0.07 enzyme units for a positive identification. Thus, from the value shown for normal rabbit serum the P/N ratio for a test sera would need to be 2.0 or higher to be considered positive.

TABLE 6. PRECISION OF ENZYME-LINKED IMMUNOASSAY FOR COXSACKIEVIRUS TYPE B-2

Serum	Replicate	Enzyme units bound	Mean units bound	Standard deviation
Normal	1	0.073		
rabbit serum	2	0.065	0.071	0.006
	3	0.076		
Anti-coxsackie virus B-2	1	0.625		
	2	0.552	0.584	0.037
	3	0.575		

For identification of selected enterovirus types (poliovirus type 1, PO-1, coxsackievirus type B1, CB-1, CB-2, and coxsackievirus type A-9, (CA-9) the adsorbed antigen method was utilized. Homotypic and heterotypic rabbit antiviral sera were used; the indicator of the reaction was peroxidase-labeled goat anti-rabbit IgG as above. The results given in Table 7 show that all of the viruses tested are positively identified when compared with normal sera, giving P/N ratios of 2.5 or higher, and all gave higher P/N ratios with homotypic than with heterotypic sera. Some of the heterotypic sera did give stronger reactions than normal sera, but with the exception of the two CB viruses, all had P/N ratios of less than 1.7. The higher values obtained with some of the heterotypic sera could indicate a degree of antigenic relatedness, but more extensive studies utilizing viral antigens of greater purity would be required before any definitive conclusions are reached. For the coxsackie B viruses, there was significant cross reaction between types B-1 and B-2. Subsequent testing of all the viruses in the B group (data not shown) indicated that these viruses could be accurately identified as to group only. Reaction of B-group viral antigens with anti-sera to virus types in other groups was minimal, as measured by either Microtiter plate assays (not shown) or by spectrophotometric tests (Table 7).

TABLE 7. REACTION OF ENTEROVIRUS TYPES WITH TYPE-SPECIFIC AND HETEROTYPIC ANTISERA BY ENZYME-LINKED IMMUNOASSAY

Viral antisera used	Enzyme units bound: Virus antigen adsorbed							
	PO-1	P/N*	CB-1	P/N	CB-2	P/N	CA-9	P/N
PO-1	0.32	2.9	0.17	1.4	0.23	1.6	0.09	1.3
CB-1	0.09	0.8	0.30	2.5	0.21	1.5	0.08	1.1
CB-2	0.17	1.5	0.25	2.0	0.47	3.4	0.10	1.5
CA-9	0.14	1.3	0.10	0.8	0.19	1.4	0.24	3.4
Normal rabbit serum	0.11	(1.0)	0.12	(1.0)	0.14	(1.0)	0.07	(1.0)

* Enzyme units bound with viral antisera (positive)/units bound with normal rabbit serum (negative).

To compare the adsorbed antigen with the adsorbed antibody method, enzyme-linked immunoassays were tested on several virus types. The results shown in Table 8 indicate that both methods gave approximately the same results for identification of echovirus type 6 (EC-6), PO-1, CB-1 and CA-9, with P/N ratios >2.0. Cell antigen preparations were tested against virus-specific antisera for all types, and gave P/N ratios varying from <1.0 to the 1.3 value shown for anti-CA-9 in Table 8.

TABLE 8. COMPARISON OF ADSORBED ANTIGEN AND ADSORBED ANTIBODY METHODS FOR ENTEROVIRUS IDENTIFICATION BY ENZYME LINKED IMMUNOASSAY

Antigen tested	Adsorbed antigen method			Adsorbed antibody method		
	Type-specific antiserum	NRS	P/N*	Type-specific antiserum	NRS	P/N
EC-6	0.28	0.08	3.5	0.27	0.12	2.3
PO-1	0.34	0.11	3.1	0.24	0.11	2.2
CB-1	0.31	0.13	2.4	0.19	0.09	2.1
CA-9	0.50	0.12	4.2	0.43	0.08	5.3

(continued)

TABLE 8. (continued)

Antigen tested	Adsorbed antigen method: Type-specific antiserum	NRS	P/N*	Adsorbed antibody method: Type-specific antiserum	NRS	P/N
cell extract†	0.15	0.11	1.3	0.10	0.08	1.3

*Enzyme units bound with viral antisera (positive)/units bound with normal rabbit sera (NRS) (negative).

†Serum used for cell extract was anti CA-9.

SENSITIVITY OF ASSAYS

The sensitivity of the assays for enteroviruses, in terms of the number of plaque-forming units (PFU) required to give a positive test, was determined for CA-9. Virus was adsorbed to polystyrene tubes at dilutions of 1:5 to 1:500 from an initial concentration of 2.0×10^7 PFU/ml for CA-9, and 5.6×10^7 PFU/ml for EC-6 which was used as a control for possible cell antibody presence in viral antisera. Rabbit anti-CA-9 sera was added at a 1:100 dilution, followed by goat anti-rabbit IgG labeled with peroxidase. The substrate used to indicate presence of bound enzyme was 0.08% w/v 5-aminosalicylic acid (5-AS) in distilled water at pH6.0 plus 0.005% H_2O_2. The absorbance at 460 nm was measured after 15 min reaction. The results in Fig. 1 show that the highest dilution of CA-9 giving A_{460} values 2.0 or more times that of either the EC-6 or cell antigen controls was between 1:200 and 1:100, which is equivalent to 1.0 to 2.0×10^5 PFU/assay tube.

A similar assay was done to determine the titer of antisera used for CA-9 identification. Virus and cell antigen was adsorbed to polystyrene tubes at a 1:10 dilution, and rabbit anti-CA-9 sera was added at dilutions from 1:50 to 1:1600. The titer of the antisera used, after absorption with cell debris, was 1:1600 as determined by plaque reduction. Serum controls tested were rabbit anti-CB-1 and normal rabbit serum. Peroxidase-labeled antiglobulin and 5-AS substrate were added as above. The results in Fig. 2 show that up to a 1:800 dilution of anti-CA-9 sera was clearly positive (P/N=2.1 from data), when compared with the control most strongly reactive.

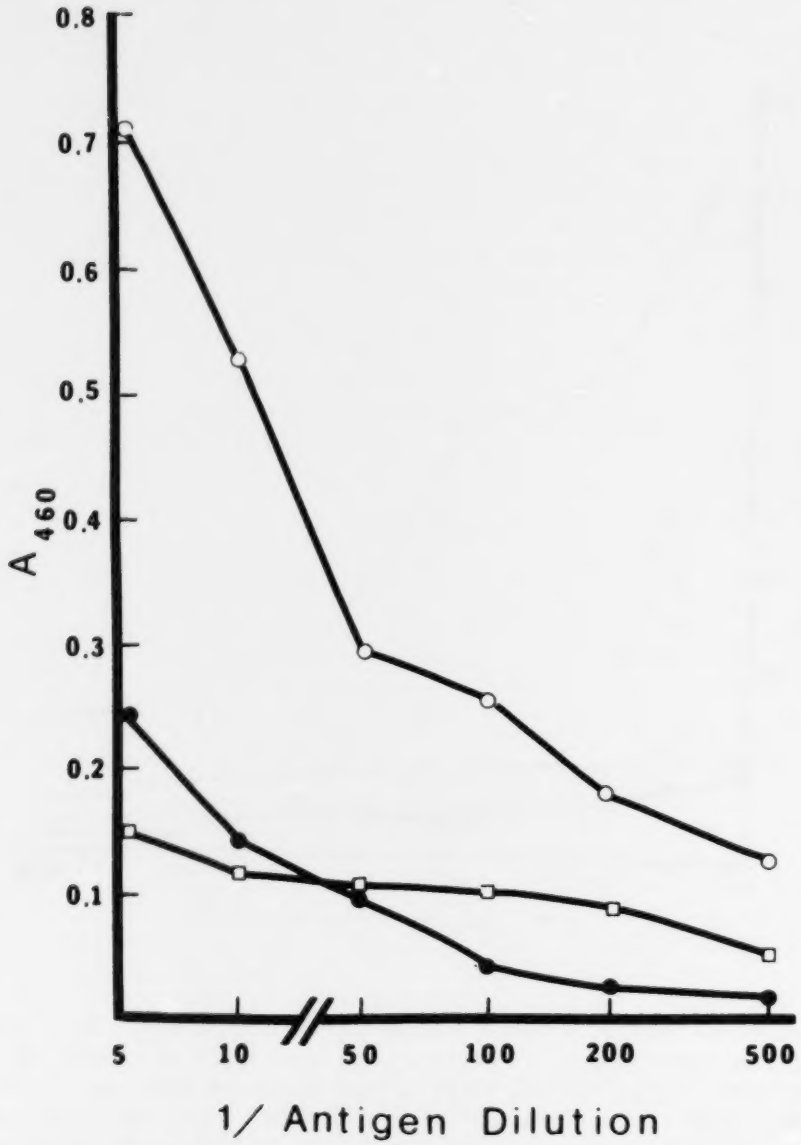

Fig. 1. Detection limits of coxsackievirus type A9 (CA-9) by enzyme-linked immunoassay. Virus concentration per sample tube at a 1:5 dilution was 4 x 10^6 PFU. Symbols (O), CA-9 antigen reacted with anti-CA-9 sera; (●), CA-9 antigen reacted with anti-echovirus type 6 sera; (□), cell antigen reacted with anti-CA-9 sera.

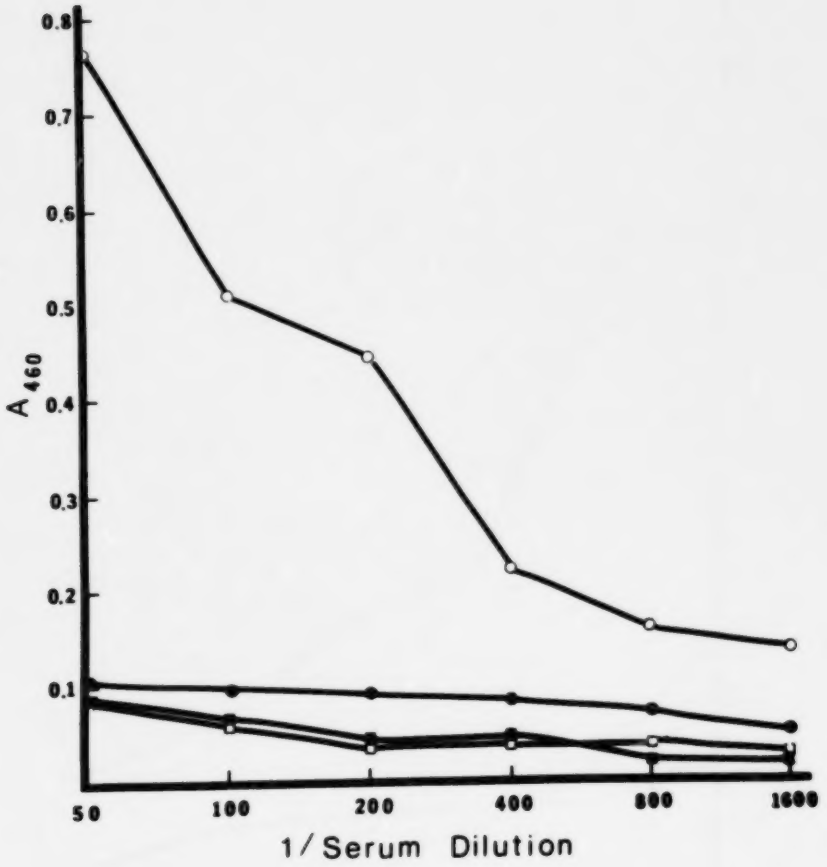

Fig. 2. Titration of antibody to coxsackievirus type A-9 (CA-9) by enzyme-linked immunoassay. CA-9 was used at a 1:10 dilution, as was cell antigen. Symbols (O), CA-9 antigen reacted with anti-CA-9 sera; (●), cell antigen reacted with anti-CA-9 sera; (□), CA-9 antigen reacted with anti-coxsackievirus type B2 sera; (■), CA-9 antigen reacted with normal sera.

IMMOBILIZED ANTIBODY FOR IMMUNOASSAY

Because of the problem of elution of virus and immunoreagents non-specifically adsorbed to plastics, we tested the effectiveness of covalently linking antibody to nylon, utilizing rabbit IgG as a model antigen. To do this, an enzyme-linked immunoassay or nylon for rabbit IgG was done. Goat anti-rabbit IgG was coupled to nylon balls through amino groups with glutaraldehyde or through carboxyl groups with CMC as described. HCl-treated nylon, which we found in preliminary experiments to adsorb antibody more readily than untreated nylon, was used as a non-coupled control. Dilutions of rabbit IgG were added; peroxidase-labelled goat anti-rabbit IgG was the indicator of the reaction.

The results in Fig. 3 show the relative sensitivity of the assay procedures. All showed a linear response over a 50-5000 ng range, with the glutaraldehyde coupled immunosorbent showing the greatest capacity for binding IgG. Antibody adsorbed to HCl-treated nylon showed a far less binding capacity than either of the covalently-linked immunosorbents. Although the purpose of the experiment was to compare relative, not absolute sensitivity, the coupled nylon gave about the same sensitivity for IgG as that reported for methods utilizing insoluble antibody or acrylamide bead immunosorbents (27). The antisera used in the above experiment and the ones to follow were not immunospecific, i.e. not purified by affinity chromatography.

A problem that has been noted for immobilized enzymes is that enzymes bound close to a polymer surface may cause a decrease in enzyme activity due to steric hindrance. To overcome this, straight-chain spacers, such as polyamino acids, have been used (15). Because there may be loss of apparent avidity of antibody covalently bound to solids, also due to steric hindrance (28,29), an immunoassay utilizing spacers was done. To test if spacers would enhance the binding capacity of goat anti-rabbit IgG immobilized on nylon, poly-L-lysine was bound to nylon balls with CMC prior to coupling antibody with glutaraldehyde. An enzyme-linked immunoassay for rabbit IgG was done in the same manner as that described above (Fig. 3). The results presented in Table 9 show that the use of poly-L-lysine as a spacer increased the total binding capacity of the immunosorbent for IgG at high concentrations. The sensitivity was about the same as that obtained by immunosorbents prepared with glutaraldehyde alone, but was more sensitive than that obtained by use of CMC alone (Fig. 3).

The relative efficiency of immunosorbents prepared by covalent coupling were higher for binding IgG, as measured by enzyme immunoassay, than non-coupled ones (Fig. 3, Table 9). To determine if the efficiency of immuno-assay was correlated with the efficiency of antibody attachment, IgA (mouse myeloma protein) labelled with ^{125}I was adjusted to 1 mg/ml with unlabelled immunoglobulin and reacted with nylon balls. The nylon balls were activated with glutaraldehyde or CMC, or were HCl-treated or untreated. After immunoglobulin attachment, the balls were washed in a solution of PBS plus 2% w/v BSA and 0.15% v/v Tween 20, and incubated further in the same solution for 3 h at room temperature, or 3 h at room temperature plus 21 h at 4°C. This solution is the one most often used for preventing non-specific

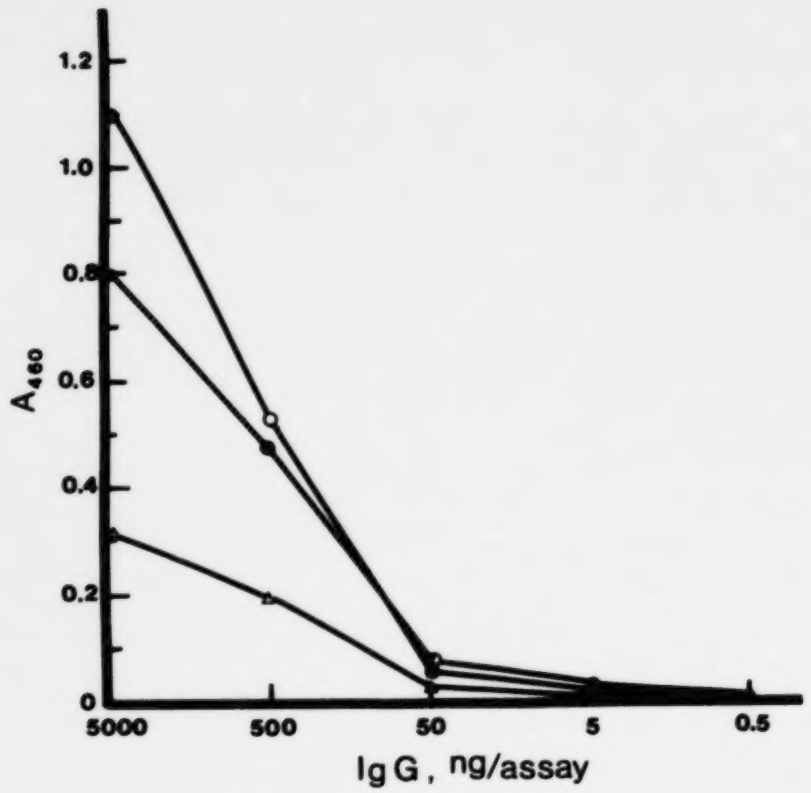

Fig. 3. Enzyme-linked immunoassay of rabbit IgG on nylon balls coupled with goat anti-rabbit IgG. (O) antiglobulin glutaraldehyde coupled; (●) antiglobulin CMC coupled; (Δ), antiglobulin adsorbed (HCl-treated nylon).

TABLE 9. ENZYME-LINKED IMMUNOASSAY OF RABBIT IgG. USE OF SPACERS FOR COUPLING GOAT ANTI-RABBIT IgG TO NYLON BALLS

Rabbit IgG assayed (ng/ml)	Glutaraldehyde		Poly-L-lysine+CMC		HCl-treated		Untreated	
	A_{460}	P/N*	A_{460}	P/N	A_{460}	P/N	A_{460}	P/N
5000	1.144	76.2	1.333	83.3	0.333	16.9	0.222	10.6
500	0.540	36.0	0.581	36.3	0.200	10.0	0.125	5.9
50	0.081	5.4	0.086	5.4	0.040	2.0	0.040	1.9
5	0.042	2.8	0.032	2.0	0.014	0.6	0.014	0.7
0†	0.015	1.0	0.016	1.0	0.020	1.0	0.021	1.0

*P/N, ratio of A_{460} IgG samples/A_{460} of bovine serum albumin control samples. A P/N $\geq$ 2.0 shows differences between test and control samples at a 95% confidence level (Mann-Whitney test).

†Bovine serum albumin, 1 mg/ml, was used as control.

adsorption in enzyme immunoassays, and, as we showed previously (Tables 3 and 4), will desorb proteins attached by simple adsorption to plastics. After incubation, the balls were washed in PBS and counted in an automatic gamma counter.

The results given in Table 10 show that little if any desorption occurred on nylon balls treated with glutaraldehyde, which was also about 15 times as effective in attaching immunoglobulin as untreated nylon. CMC-coupled and HCl-treated nylon balls gave intermediate values, all of which correlated well with the results shown in Fig. 3. Because desorption of antibody from the surface of a solid-phase carrier can bind free antigen and thus lower the sensitivity of an immunoassay, the advantage of covalent coupling is apparent.

TABLE 10. EFFICIENCY OF ATTACHMENT OF ^{125}I-LABELED IgA TO NYLON BALLS

Desorption time* (h)	IgA bound (%)†			
	Nylon coupling agent or nylon treatment			
	Glutaraldehyde	CMC	HCl	None
0	20.9	15.1	11.5	3.1
3	21.0	10.3	9.3	1.5
24	18.9	9.3	7.3	1.3

*Time treated with phosphate buffered saline with 2% w/v bovine serum albumin and 0.15% v/v Tween-20 added.

†^{125}I-labelled IgA remaining/total ^{125}I-labelled IgA input, X 100.

EFFICIENCY OF IMMOBILIZATION

The relative amount of immunoglobulin (Ig) that can be immobilized on various plastics was also determined. The amount of IgG in $\mu g/mm^2$ that could be immobilized on nylon (Table 11) was 37 times the amount which could be immobilized on polystyrene, the plastic most often used for ELISA tests, or on poly-methyl-methacrylate (PMMA), a plastic used in some types of Microtiter plates.

TABLE 11 COMPARISON OF METHODS FOR IMMOBILIZING IgG ON PLASTICS

Plastic	Treatment	Desorption time*			
		None		24 hours	
		Percent bound	μg IgG/mm^2	Percent bound	μg IgG/mm^2
Nylon	Poly-L-lysine+CMC	31.7	0.98	23.9	0.78
	Glutaraldehyde	20.9	0.65	18.9	0.59
	CMC	15.1	0.47	10.3	0.32
	HCl	11.3	0.35	7.3	0.23
	None	2.5	0.08	1.3	0.04

(continued)

TABLE 11 (continued)

Plastic	Treatment	Desorption time*			
		None		24 hours	
		Percent bound	µg IgG/mm^2	Percent bound	µg IgG/mm^2
PMMA	None	1.1	0.03	0.8	0.02
Polystyrene	None	0.7	0.02	0.7	0.02

*Time treated with phosphate-buffered saline with 2% w/v bovine serum

†^{125}I-labelled Ig remaining/total ^{125}I-labelled Ig input, X 100.

RE-USE OF IMMOBILIZED ANTIBODY

Another advantage of covalent coupling of antibodies to insoluble carriers is the potential for antibody re-use. This was tested for goat anti-rabbit IgG coupled (glutaraldehyde method) to nylon balls. An enzyme-linked immunoassay was done for rabbit IgG with peroxidase-labelled goat anti-rabbit IgG as the indicator of the reaction, as above. After the results were recorded forthe initial reaction (Table 12), the balls were regenerated by treatment with low pH buffers, as indicated in the table. Between assays, enzyme substrate was added to the regenerated balls to insure that no residual enzyme activity remained. Once this was established, the immunosorbents were tested for IgG binding as before (data not shown), regerated and re-assayed. The results given in Table 12 show that both the A_{460} and P/N values obtained were approximately the same at all IgG concentrations tested, indicating that covalently coupled antibody remains bound to the nylon used, and the bound antibody is suitable for re-use at least two times.

TABLE 12. RE-USE OF NYLON BALLS COUPLED WITH GOAT ANTI-RABBIT IgG FOR ENZYME LINKED IMMUNOASSAY

Rabbit IgG assayed (ng/ml)	Enzyme-linked immunoassay of rabbit IgG					
	Initial assay		Treatment prior to re-assay			
			HCl-NaCl*		Glycine-HCl†	
	A_{460}	P/N‡	A_{460}	P/N	A_{460}	P/N
5,000	1.269	84.6	1.161	82.9	1.210	80.7
500	0.599	39.9	0.537	38.4	0.557	37.1
50	0.111	7.4	0.103	7.4	0.098	6.5
5	0.045	3.0	0.057	4.1	0.026	1.7
0§	0.015	1.0	0.014	1.0	0.015	1.0

*Nylon treated for 0.5 h with 0.003M HCl in 1 M NaCl, and washed in phosphate-buffered saline.

†Nylon treated for 0.5 h with 0.1M glycine-HCl (pH 2.2), rinsed in 1 M NaCl, and washed in phosphate-buffered saline.

‡P/N, ratio of A_{460} of IgG samples/A_{460} of bovine serum albumin control samples.

§Bovine serum albumin, 1 mg/ml, was used as control.

IMMUNOASSAY ON NYLON POWDER

The use of nylon balls provides a convenient solid-phase for performing enzyme-linked immunoassays, and the washing procedures require no centrifugation. The use of nylon powder, though, should provide more surface area per volume or weight of nylon, and thus be a more efficient immunosorbent.

To test the usefulness of nylon powder for antibody immobilization, an enzyme-linked immunoassay of rabbit IgG was done. Approximately 0.5ml of activated, packed nylon powder was washed in PBS and suspended in 0.5ml of an IgG fraction of goat anti-rabbit IgG. After 2 h at room temperature, 0.5ml of PBS was added and the mixture incubated for 18-24 h at 4°C. For assay, the antibody-coupled powder was washed 3 times in PBS by centrifugation at 3500 x g, and suspended 1:5 in PBS plus 2% w/v BSA and 0.05% Tween 20. A 0.02ml amount of this suspension was added to 0.5ml of rabbit IgG, diluted in PBS containing 10% v/v normal goat serum, and incubated 1 h at 37°C. The procedures from this point on were the same as that described for immunoassay on nylon balls, except that washing procedures were done by centrifugation. Peroxidase-labelled goat anti-rabbit IgG was the indicator used. The results given

in Table 13 show that the sensitivity of enzyme-linked immunoassay on nylon powder was approximately that obtained by use of nylon balls, with low background values for control samples (normal goat sera).

TABLE 13. ENZYME-LINKED IMMUNOASSAY OF RABBIT IgG IMMOBILIZED ON NYLON POWDER

Rabbit IgG assayed (ng/ml)	Nylon powder treatment			
	Glutaraldehyde		None	
	A_{460}	P/N*	A_{460}	P/N
5000	0.735	91.9	0.478	9.3
500	0.433	54.1	0.181	3.5
50	0.045	5.6	0.061	1.2
5	0.014	1.8	0.035	0.7
Normal goat serum	0.008	1	0.051	1

*P/N, ratio of A_{460} IgG samples/A_{460} of normal goat serum control samples.

BINDING OF ENTEROVIRUSES TO ACTIVATED NYLON.

Direct coupling of virus to activated nylon was tested with ^{14}C-leucine labelled poliovirus 2 (PO-2) and coxsackievirus B3 (CB-3). Dilutions of each, 1:10 in PBS, were added to untreated nylon or glutaraldehyde-activated nylon balls (0.5 ml/6 balls), incubated and treated with BSA as for the IgG experiments above, and washed. The amount of virus remaining was determined by counting ^{14}C disintegration in a liquid scintillation spectrometer. The results in Table 14 show that the coupling procedure was about two to three times as effective in attaching virus than that obtained by simple non-specific adsorption (untreated nylon or HCl-treated nylon).

TABLE 14. COUPLING OF ENTEROVIRUSES TO NYLON BALLS

Virus added	Virus bound (%)		
	Activated nylon	HCl-treated nylon	Untreated nylon
^{14}C-labelled PO-2	44.0	18.0	10.0
^{14}C-labelled CB-3	45.0	22.0	7.6

There was also more virus bound than was obtained by simple adsorption to polystyrene (Table 4), although the results aren't directly comparable.

ENZYME-LINKED IMMUNOASSAY OF ENTEROVIRUSES ON NYLON

Assay of enteroviruses by use of activated nylon was done by a direct and indirect method. Specific enterovirus antisera (rabbit) or control sera was added, followed by goat anti-rabbit IgG labelled with peroxidase. Enzyme substrate was added, and the product read at 460 mm after 10 min. For the indirect test, specific horse antiviral sera was coupled to nylon balls as described for antibody rabbit IgG above. These were incubated with virus, and rabbit antiviral sera added for specific identification as in the direct test. The results are shown in Table 15. By the direct method, poliovirus 2 (PO-2) and poliovirus 3 (PO-3) could be identified, and the test could distinguish them from the other poliovirus types. By the indirect test, PO-2 gave a strong reaction compared with coxsackievirus type B2 (CB-2), and PO-3 was distinguished from poliovirus 1 (PO-1). In both cases, the use of chemical coupling methods gave better results than the adsorption methods used in the conventional ELISA tests, especially for the coupled antibody method. However, it was not possible to obtain as low background (normal sera) values as those obtained for the experiments utilizing rabbit IgG as a model antigen.

TABLE 15. ENZYME IMMUNOASSAY OF ENTEROVIRUSES ON ACTIVATED NYLON

Method	Virus Type	Antisera	Enzyme Units Bound	P/N*
Direct†	PO-2	PO-2	0.24	3.0
		PO-1	0.075	0.9
		NRS	0.08	1.0
	PO-3	PO-3	0.26	2.6
		PO-2	0.16	1.6
		NRS	0.10	1.0
Indirect‡	PO-2	PO-2	0.49	6.1
		CB-2	0.11	1.4
		NRS	0.08	1.0

Legend (continued)

*P/N, ratio of enzyme units bound with viral antisera/units bound with normal rabbit sera (NRS).

†Virus coupled to nylon.

‡Viral antisera coupled to nylon.

SECTION 6

DISCUSSION

The enzyme-linked immunoassay technique used was found suitable for type specific identification of the enterovirus types selected, and for group identification of coxsackie B viruses. The reason for the lack of specificity within the B group is not clear, but may be related to the viral antigens that adsorb most strongly to the plastic, and/or to the antisera used. Group antigens have been demonstrated in this group (3), which could adsorb more strongly, and for diagnosis of coxsackie B viruses by immunofluorescence, neither horse nor rabbit antisera were found entirely satisfactory for type-specific identification, whereas hamster antisera and mouse immune ascitic fluid were (8).

Because of the low amount of viral antigen present in enterovirus preparations (ca. 1 ng/10^8 virions) and because some of the types are antigenically related, immunoassay of these viruses requires optimal conditions. It was found that for direct adsorption of viral antigen to polystyrene tubes or plates, some type of partial purification was necessary. Extraction of the virus samples with ether or a batch-method treatment with an anion-exchange resin removed sufficient cellular protein to permit adsorption, although maximum adsorption was obtained with virus purified by passage through a column of DEAE-Sephadex A-50. For routine diagnostic work the simpler methods are sufficient, and no gain in accuracy was obtained by use of purified virus (data not given).

The precision of the assay was high, when a given set of reagents were tested under the same conditions. Over a period of time, when different reagents are used, the precision would undoubtedly be less, although our background values for normal sera were quite similar over periods of several months. Most reports consider a positive/negative ratio of 1.8:1 or more to indicate a positive result in an immunoassay. The ratio selected, though, depends on the precision of an individual set of assays, as there is not as yet a standard method. Increasing the precision can be done by decreasing non-specific adsorption; we found that use of PBS plus 2% BSA and 0.15% Tween 20 for diluting immunoreagents gave the lowest background values. Others have found that PBS plus 0.05% Tween 20 is sufficient for inhibiting non-specific reactions (2). In our study, use of this diluent permitted 0.28 units of enzyme conjugate to adsorb to polystyrene cuvettes, which is as high a number as some of our positive tests. However, the disadvantage of using diluents with inhibitors is that desorption of antigen and antibody during an immunoassay is increased. We found that for adsorbed viral antigen,

a high percentage of virus elutes when incubated with the diluents used for immunoassay. Desorption of adsorbed antibody was minimal at concentrations of 2 and 10 µg/ml. Engvall, et al. (31), however, found that desorption of antibody was about 40% for antibody adsorbed at 2 µg/ml and incubated with PBS plus 0.05% Tween 20 alone. For these reasons, methods utilizing covalent linking of antibodies and viral antigens to nylon were developed.

By use of rabbit IgG as a model antigen, enzyme-linked immunoassays utilizing antibodies coupled to nylon balls were found to be more effective than immunoassays utilizing antibodies adsorbed to polystyrene, but were as easy to perform as the conventional ELISA tests. The effectiveness of this method is due primarily to the greater amount of antibody which can be immobilized on activated nylon, and the lack of antibody desorption during immunoassay procedures. The amount of antibody which could be immobilized per mm^2 nylon was 0.74µg, which was 37 times the amount that could be adsorbed to polystyrene balls of the same diameter. The amount that could be immobilized compares favorably with other types of non-porous supports utilizing covalent linkage as well. From the data given by Genung and Hsu (32) and by Quash, et al. (33) for covalent linkage of antibody to polystyrene latex spheres, it can be calculated that these methods were capable of immobilizing up to 0.0035µg/mm^2 and 0.1µg/mm^2 latex respectively.

Activated nylon powder was also useful for immobilization of antibody, as determined by enzyme-linked immunoassay of rabbit IgG. The surface area per weight of nylon is greater than that for nylon balls, which should allow for comparably greater quantities of antibody to be immobilized. It is not as convenient for enzyme-linked immunoassays as the nylon balls because centrifugation is required for washing samples. It was not tested for use in virus identification, but has the potential for use as an immunosorbent in concentrating virus.

For enterovirus identification by conventional ELISA, the adsorbed antigen method was found to be approximately as sensitive as the adsorbed antibody method. Both methods are satisfactory and, as the adsorbed antibody method has been used for detection of herpesvirus at low concentrations (6), it is more suitable as a virus detection method, especially if specific antisera of high titer from two species of animals are available. For enzyme-linked immunoassay on nylon, the coupled antibody method gave excellent P/N values, and because more antibody can be immobilized than by methods based on adsorption, should be more sensitive as well. This has yet to be tested. The sensitivity of the conventional ELISA method, in terms of virus concentration required for identification, was approximately 10^5 FPU of virus per assay tube. This is 10^2 to 10^3 times higher than the amount of virus required for identification by neutralization tests, but because the quantity of virus needed for ELISA is readily obtainable in fluids from infected tissue cultures, this is not considered to be a serious disadvantage of the method. Also, the need for virus titration is not necessary for ELISA, which is an advantage over neutralization tests. The dilution endpoint of antisera that could effectively be used for ELISA was approximately that obtained by neutralization tests.

The work reported here utilized individual typing sera to identify

selected enterovirus types, for developmental purposes. At the present stage of development, the method would be useful for presumptive identification of group B coxsackieviruses in situations where clinical or other evidence suggests involvement of these viruses, as an alternate means of confirming a type-specific identification of an enterovirus isolate, or as a means to verify the identity of laboratory strains, but not for direct identification of virus in water. If the method is to be useful for identification of field isolates, which could be any of a large number of enterovirus types, the use of pooled sera in an intersecting serum scheme (34) would be needed for rapid typing by any form of enzyme-linked immunoassay. Preliminary data not reported here indicate that some virus types can be identified by use of pooled sera whereas others cannot, due to cross-reaction, which is a current limitation of the method. The reasons for these cross-reactions and possible means to eliminate them need to be investigated.

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